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Award Number: DAMD17-01-1-0455

TITLE: DNA Vaccination to Overcome Peripheral Tolerance

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REPORT DATE: August 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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20041215 024

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> August 2004	<b>3. REPORT TYPE AND DATES COVERED</b> Final (1 Aug 2001 - 31 Jul 2004)	
<b>4. TITLE AND SUBTITLE</b> DNA Vaccination to Overcome Peripheral Tolerance			<b>5. FUNDING NUMBERS</b> DAMD17-01-1-0455	
<b>6. AUTHOR(S)</b> Wei-Zen Wei, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Wayne State University Detroit, Michigan 48202  <i>E-Mail:</i> weiw@karmanos.org			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited			<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b> The goal of the project is to develop vaccination regimen to a tumor associated self-antigen, ErbB-2 and to induce significant anti-tumor immunity in mice which are tolerant to ErbB-2. The objectives are to (1) Measure vaccination efficacy after depletion of CD4+CD25+ regulatory T cells. (2) Compare immune reactivity and tumor growth inhibition in ErbB-2 transgenic mice vaccinated with autologous or heterologous ErbB-2. (3) Construct and test vaccination efficacy of ErbB-2 containing Pan DR Reactive Epitope (PADRE).  Toward objective 3, we have constructed pPADRE-SecE2, defined the biochemical properties of PADRE secE2 protein and established the superior vaccination efficacy of pPADRE-SecE2 in ErbB-2 tolerant Her-2 Tg mice when compared wit pSecE2.				
<b>14. SUBJECT TERMS</b> No subject terms provided.			<b>15. NUMBER OF PAGES</b> 24	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

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## INTRODUCTION

The goal is to develop vaccination regimen to a tumor-associated self-antigen, ErbB-2 and to induce significant anti-tumor immunity in mice which are tolerant to ErbB-2. The objectives are to

- (1) Measure vaccination efficacy after depletion of CD4<sup>+</sup>CD25<sup>+</sup> cells regulatory T (Treg) cells.
- (2) Compare immune reactivity and tumor growth inhibition in ErbB-2 transgenic mice vaccinated with autologous or heterologous ErbB-2
- (3) Construct and test vaccination efficacy of ErbB-2 containing Pan DR Reactive Epitope (PADRE).

## BODY

### **Objective 3 Construct and test vaccination efficacy of ErbB-2 containing Pan DR Reactive Epitope (PADRE).**

To generate pPADRE-E2 and pPADRE-secE2 with PADRE sequence inserted between a.a. 5-6 of ErbB-2, DNA sequence encoding the signal peptide and the first 5 amino acids of human ErbB2 was isolated from pCMV E2 and cloned into KpnI/SmaI of the multiple cloning region in pUC19. The sequence encoding amino acids 6 to 70 of ErbB-2 was cloned into XbaI/AccI site downstream in the same multiple cloning region, leaving a unique BamHI restriction site between amino acids 5 and 6. Following cleavage with BamHI, the double-stranded oligonucleotide encoding PADRE, GATCCGCCAAGTTCGTGGCTGCCTGGACCCTGAAGGCT-GCCGCTAAG, was ligated into the BamHI site (1). The MluI/EcoRV fragment encoding ErbB-2 sequence with a PADRE insert was used to replace the equivalent sequence in pCMV E2 and encode the full-length transmembrane ErbB-2 with a chimeric ECD containing the PADRE peptide sequence. The MluI/BstBI fragment from pPADRE-E2 was used to replace the equivalent sequence in pSecE2 (2) to generate pPADRE-SecE2 encoding the N-terminal 505 a.a. of ErbB-2 extracellular domain containing PADRE between a.a. 5 and 6 (Figure 1A).

The biochemical and immunological properties of pSecE2 and pPADRE SecE2 were compared. C57BL/6 lung cancer cell line TC-1 cells (3) were stably transfected with psecE2 or pPADRE-SecE2. Expression of intracellular recombinant protein was measured by flow cytometry using anti-Her-2 mAb (Ab-2, Neomarkers) and goat anti-mouse Ig-PE after the cells had been fixed and permeablized (Figure 1B). Both pSecE2 and pPADRE-SecE2 transfected cells were recognized by anti-ErbB-2 mAb, demonstrating the expression of the designated recombinant protein. Expression of the SecE2 and PADRE-SecE2 was verified by Western blotting (Figure 1C). PADRE-SecE2 migrates slower than SecE2 as expected from the inserted PADRE peptide and flanking sequence. Although equivalent quantity of secE2 and PADRE-SecE2 was detected in cell lysate, very little PADRE-SecE2 was detected in the culture supernatant. Therefore, insertion of PADRE peptide between a.a. 5-6 changed the traffic of the recombinant protein and it is poorly secreted.

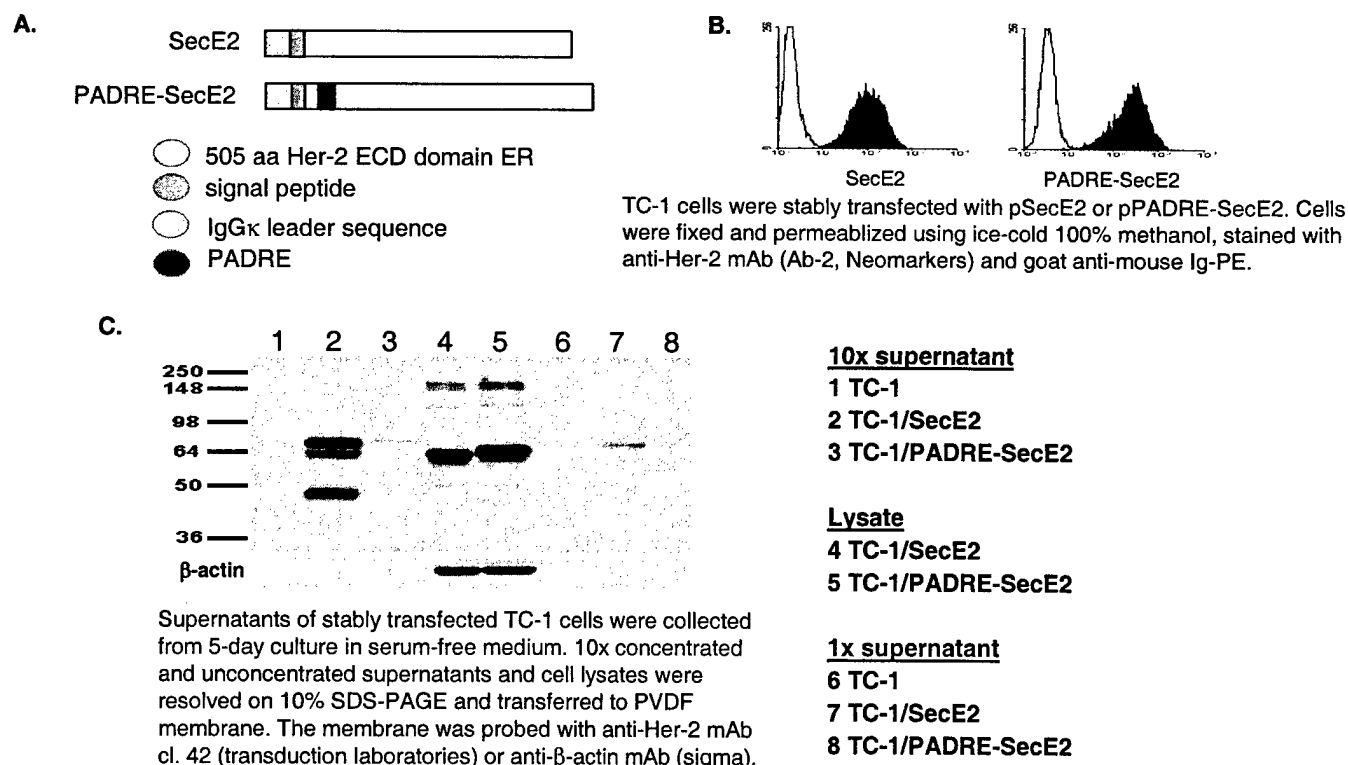
To test if anti-ErbB-2 immunity was induced with the chimeric DNA, Her-2 Tg or C57BL/6 mice were immunized with pSecE2 or pPADRE-SecE2 and anti-Her-2 Ig was measured at 2 wk after the last immunization (Figure 2). As we previously reported,

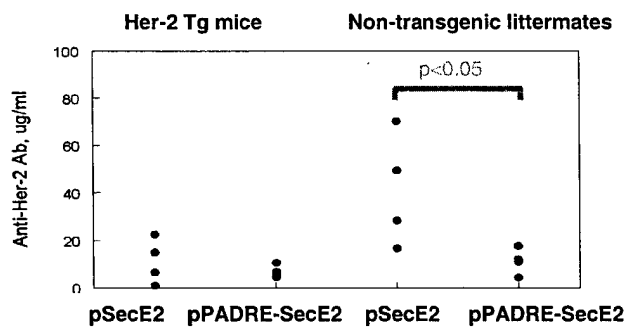
good antibody response was induced by pSecE2 in C57BL/6 mice. The same vaccine also induced anti-ErbB-2 antibody in Her-2 Tg mice, but at a much lower level as a result of their tolerance to human ErbB-2. Immunization with pPADRE-SecE2 resulted in a lower level of anti-ErbB-2 antibody in either Her-2 Tg or C57BL/6 mice, consistent with its poor secretion.

T cell response to PADRE and ErbB-2 was assessed by ELISPOT assay (Figure 3). Splenocytes were prepared from mice immunized with pPADRE-SecE2 or control vector and incubated for 24-48 hrs with 30  $\mu$ g/ml PADRE in ELISPOT plate which was pre-coated with anti-IFN- $\gamma$  antibody. About 300 spots/ 300,000 splenocytes were detected in immunized mice, demonstrating the processing and presentation of PADRE from PADRE-secE2 encoded by the DNA vaccine. To access T cell response to ErbB-2, it was necessary to generate a C57BL/6 antigen presenting cell. TC-1 cells which expressed MHC I and CD80 constitutively were transfected with human ErbB-2 to generate TC-1/E2 (Figure 4). High level of ErbB-2 specific, IFN- $\gamma$  producing T cells were detected in C57BL/6 mice which were immunized with either psecE2 or pPADRE secE2, demonstrating the processing and presentation of ErbB-2 epitopes (Figure 5). T cell response to PADRE-SecE2 in Her-2 transgenic mice is being tested.

To determine the efficacy of DNA vaccines, Her-2 transgenic mice were immunized with psecE2 or pPADRE-SecE2 together with pCD40LT and challenged with EL4/E2 cells which express human ErbB-2. Tumors were rejected in 1/4 and 3/4 mice immunized with psecE2 and pPADRE-SecE2, respectively, demonstrating greater anti-tumor immunity in ErbB-2 tolerant mice by pPADRE-SecE2 vaccination (Figure 6).

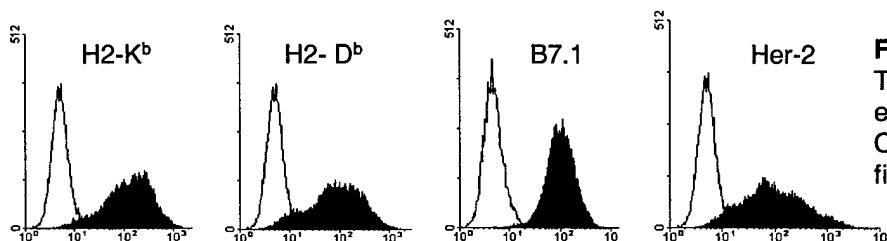
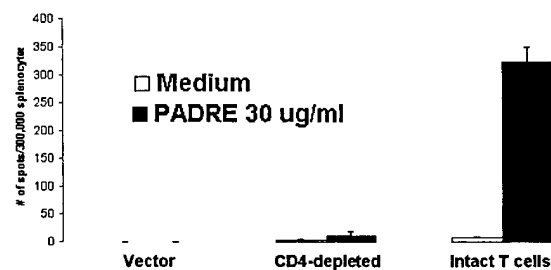
**Figure 1 psecE2 vs pPADRE-secE2**



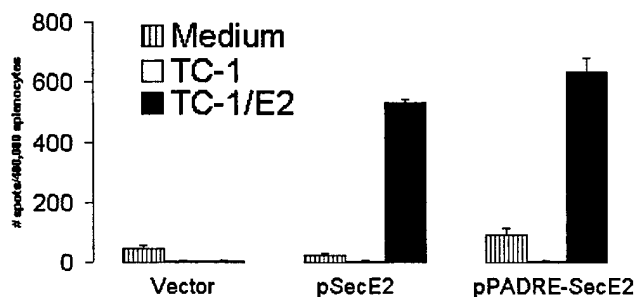


**Figure 2 Induction of anti-Her-2 Ab.** Her-2 Tg females and C57BL/6 littermates were immunized i.m. with electroporation 3X at 2wk intervals with 50ug each of pCD40LT and pSecE2 or pPADRE-SecE2. At 2 wk after final immunization, anti-Her-2 Ig was measured by flow cytometry.

**Figure 3 Induction of PADRE-specific CD4 T cells with pPADRE-secE2.** C57BL/6 mice were immunized i.m. with electroporation 3X at 3wk interval with 50ug of pPADRE-SecE2 or control vector. At 2 wk after the final immunization, IFN- $\gamma$  producing ELISPOT assay was performed with 30  $\mu$ g/ml synthetic PADRE peptide. CD4 T cells were depleted from splenocytes using anti-CD4 mAb GK1.5 and rabbit complement. Depletion efficiency was 99%.

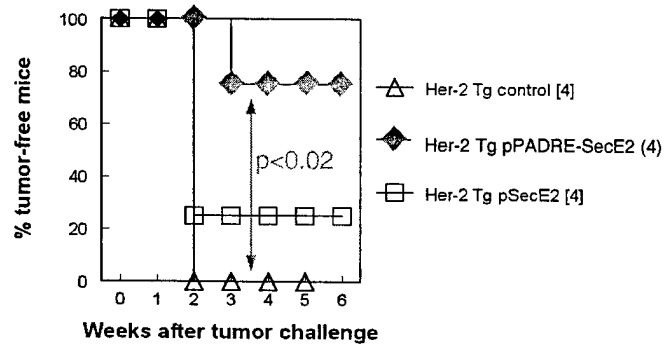


**Figure 4. TC-1/E2 antigen presenting cells.** TC-1 cells transfected with human ErbB-2 express MHC class I, CD80/B7.1 and ErbB-2. Open histogram represents control Ig stain, filled histogram represents specific Ig stain.



**Figure 5. Induction of Her-2 specific T cells with pSecE2 and pPADRE-SecE2.** C57BL/6 females (n=6) were immunized i.m. with electroporation two times at 2wk interval with 50  $\mu$ g of pSecE2, pPADRE-SecE2 or control vector. At 2 wk after the final immunization, ELISPOT assay was tested against TC-1 or TC-1/E2.

**Figure 6. Vaccination efficacy of pSecE2 and pADRE-SecE2 in Her-2 Tg mice.** Her-2 Tg females were immunized three times with 50 µg of pADRE-SecE2, pSecE2 or control vector together with 50 µg of pCD40LT. At 2wks after final immunization, mice were challenged with  $2 \times 10^5$  EL4/E2. Tumor growth was monitored by weekly palpation.



## KEY RESEARCH ACCOMPLISHMENTS

1. Construct pADRE-SecE2 and define the biochemical properties of the PADRE secE2 protein
2. Establish the superior vaccination efficacy of pADRE-SecE2 than pSecE2 in ErbB-2 tolerant Her-2 Tg mice.

## REPORTABLE OUTCOMES

Manuscripts cited in the last progress report as “in press” have been published.

Olga Radkevich, Marie Piechocki, Shari Pilon, David Shim, Richard F. Jones, and Wei-Zen Wei Protection of human ErbB-2 transgenic mice from tumor growth by vaccination, following regulatory T cell depletion, with DNA encoding Her-2 fused to exogenous antigenic sequences. Proc. AACR, 2004

Marie P. Piechocki, Ye-Shih Ho, Shari Pilon and Wei-Zen Wei, Human ErbB-2 (Her-2) transgenic mice: A model system for testing Her-2 based vaccines, J. Immunol. 171: 5787-5794, 2003

Wei-Zen Wei, Gerald P. Morris and Yi-chi M. Kong, Anti-tumor immunity and autoimmunity: a balancing act of regulatory T cells. Cancer Immunology and Immunotherapy, 53:73-78, 2004

## CONCLUSIONS

Toward Aim 3, pADRE-SecE2 has been constructed and the biochemical properties of the recombinant protein have been characterized. This plasmid demonstrated superior vaccination efficacy in ErbB-2 tolerant Her-2 Tg mice. The anti-ErbB-2 T cell immunity in Her-2 Tg mice is still being examined due to the limited number of Her-2 Tg mice we could produce. A one-year no-cost extension is requested to complete the immunological analysis of pADRE-SecE2 vaccine in Her-2 Tg mice.

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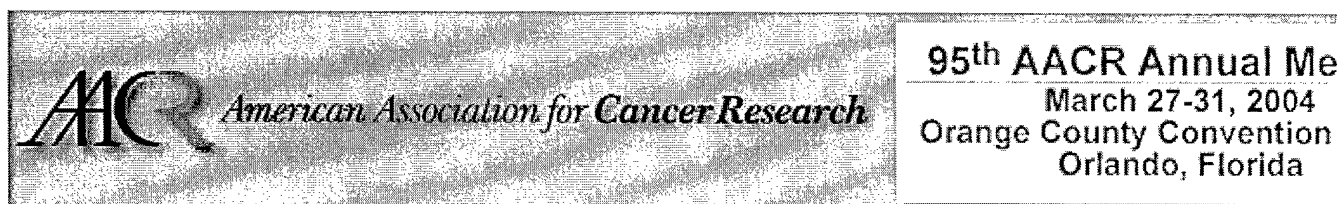
## APPENDICES

Olga Radkevich, Marie Piechocki, Shari Pilon, David Shim, Richard F. Jones, and Wei-Zen Wei. Protection of human ErbB-2 transgenic mice from tumor growth by vaccination, following regulatory T cell depletion, with DNA encoding Her-2 fused to exogenous antigenic sequences. *Proc. AACR*, 2004

Marie P. Piechocki, Ye-Shih Ho, Shari Pilon and Wei-Zen Wei, Human ErbB-2 (Her-2) transgenic mice: A model system for testing Her-2 based vaccines, *J. Immunol.* 171: 5787-5794, 2003

Wei-Zen Wei, Gerald P. Morris and Yi-chi M. Kong, Anti-tumor immunity and autoimmunity: a balancing act of regulatory T cells. *Cancer Immunology and Immunotherapy*, 53:73-78, 2004





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**5486 Protection of human ErbB-2 transgenic mice from tumor growth by vaccination, following regulatory T cell depletion, with Her-2 DNA fused to exogenous antigenic sequences.**

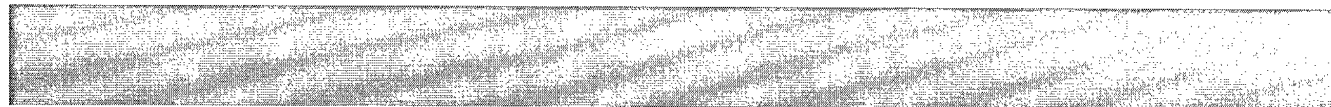
Olga ■Radkevich■, Marie P. Piechocki, Shari Pilon, David Shim, Richard F. Jones, Wei-Zen Wei. *Karmanos Cancer Institute, Wayne State University, Detroit, MI and State University of New York, Upstate Medical School, Syracuse, NY.*

To test the efficacy of Her-2 vaccines in tolerant hosts, we generated human ErbB-2 (E2) transgenic (Her-2 Tg) mice and are testing a panel of DNA vaccines encoding Her-2 containing exogenous peptides. Transgenic mice express Her-2 under whey acidic protein (WAP) promoter and have been backcrossed to C57Bl/6 for >12 generations (Piechocki, et al., J. Imm. In press). These mice demonstrate tolerance to Her-2 which can be partially overcome by DNA vaccination. When they were challenged with Her-2 expressing EL4/E2 tumor cells after five i.m. injections with DNA encoding secreted extracellular domain (ECD) of Her-2 (secE2), 33% of the mice were protected, while all immunized non-Tg littermates rejected the tumor. To test if exogenous antigenic peptide could increase the immunogenicity of Her-2 vaccine, Pan DR reactive epitope (PADRE) was inserted between a.a. 5-6 in secE2 to generate secE2-PADRE. Intramuscular injection of Her-2 Tg mice, 5 times, with secE2-PADRE and secE2 DNA protected 3/4 mice and 1/4 mice, respectively. Therefore, co-expression of PADRE may enhance anti-Her-2 immunity in tolerant mice. To test if depletion of CD4+25+ regulatory T (Treg) cells could release anti-Her-2 effectors from negative regulation, mice were treated with anti-CD25 mAb PC61 on days 5 and 6 before they received secE2 and GM-CSF DNA via i.m. injection followed by electroporation. In 90% of Treg depleted Her-2 Tg mice, EL4/E2 tumor challenge was rejected after three vaccinations. In the absence of Treg depletion, the same vaccine protected only ~ 30% of mice. To assess anti-Her-2 response, anti-Her-2 antibody was measured by flow cytometry. Consistent with greater inhibition of tumor growth afforded by Treg cell depletion, elevated anti-Her-2 antibody level was detected in Treg cell depleted group twice immunized with secE2 and GM-CSF DNA ( $40.8 \pm 19.2$  ug/ml) versus non-depleted group immunized twice ( $14.5 \pm 11.5$  ug/ml) or three times ( $21.7 \pm 23.2$  ug/ml). Therefore, tolerance in Her-2 Tg mice was overcome by i.m. injection and electroporation of DNA encoding secE2 following Treg cell depletion. Additional Her-2 DNA constructs have been generated. Tetanus toxoid fragment C (tetC) was fused to the C-terminus of secE2 as secE2-tetC. Domain 1 of tetC (Td1) was fused to the C-terminus of Her-2 fragments as E2ECD-Td1 and E2TM-Td1. The efficacy of these

fusion vaccines is being evaluated and compared with secE2-PADRE. This study is supported by CA76340 and DAMD17-01-1-0455.

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Citation information: Proceedings of the AACR, Volume 45, March 2004.



# Human ErbB-2 (Her-2) Transgenic Mice: A Model System for Testing Her-2 Based Vaccines<sup>1</sup>

Marie P. Piechocki,<sup>\*†</sup> Ye-Shih Ho,<sup>§</sup> Shari Pilon,<sup>\*‡</sup> and Wei-Zen Wei<sup>2\*‡</sup>

Her-2 transgenic (Tg) mice were generated with wild-type human c-ErbB-2 (Her-2) under the whey acidic protein promoter. They are tolerant to Her-2 and appropriate for testing Her-2 vaccines. The expression of transmembrane ErbB-2 from the whey acidic protein-Her-2 cassette and its up-regulation by insulin and hydrocortisone was verified by *in vitro* transfection. The transgene cassette was microinjected into fertilized eggs from B6C3 (C3H × C57BL/6) females mated with B6C3 males. Transgene-positive mice were backcrossed onto C57BL/6 mice. Human ErbB-2 was expressed in the secretory mammary epithelia during pregnancy and lactation and expressed constitutively in the Bergman glia cells within the molecular layer of the cerebellum. Overt, neoplastic transformation was not detected in any tissue examined. Tolerance to Her-2 was demonstrated by inoculating mice with a syngenic tumor expressing high levels of human ErbB-2. Tumors grew exclusively in Her-2 Tg mice without inducing an Ab response, while the nontransgenic littermates remained tumor free for 10 mo and mounted a robust anti-ErbB-2 Ab response. When immunized five times with plasmid DNA encoding secErbB-2 and GM-CSF, respectively, ~33% of the Her-2 Tg mice rejected a lethal challenge of EL-4/E2 tumor cells, whereas all immunized littermates rejected the tumor. Therefore, Her-2 Tg mice express human ErbB-2 in the brain and mammary gland and demonstrated tolerance to ErbB-2 which was partially overcome by DNA vaccination. The breakable tolerance of Her-2 Tg mice resembles that in human and these mice are particularly suited for testing human ErbB-2 based vaccines. *The Journal of Immunology*, 2003, 171: 5787–5794.

**E**rbB-2 is amplified in ~30% of all breast cancers and is over-expressed in several other epithelial-derived neoplasms including ovarian cancer, small cell lung cancer, and cancers of the head and neck (1–3). The presence of ErbB-2 specific T cells and Abs in breast and ovarian cancer patients indicated this molecule as a target of immunoprevention and therapy (4–7). Anti-ErbB-2 mAb, Herceptin, is used to treat patients with advanced breast cancer (8). We have generated several human ErbB-2 (Her-2) based DNA vaccines and demonstrated striking anti-tumor immunity in mice (9–11). Since ErbB-2 is a self Ag and the sequence is typically unmodified in human cancer, immune tolerance to this tumor associated Ag is expected in humans. Therefore, the efficacy of ErbB-2 based immunotherapy or vaccines will have to be tested in tolerant hosts.

Several rat ErbB-2 (*neu*) transgenic (Tg)<sup>3</sup> mouse strains have been established. FVB-NeuN mice carry wild-type rat *neu* driven by the mouse mammary tumor virus (MMTV) promoter in the H-2<sup>a</sup> FVB background. Overexpression of the transgene in the mammary gland results in mammary tumor growth around 40 wk of age (12). BALB NeuT mice carry activated rat *neu* oncogene driven by the MMTV promoter in the H-2<sup>d</sup> BALB/c background

(13). Spontaneous mammary tumors appear in BALB NeuT females around 20 wk of age. Although these models are interesting and useful, there is ~10% difference between rat and human ErbB-2 proteins (14). When immunized with human ErbB-2 DNA, FVB-NeuN females developed less tumors, but neither humoral nor cellular immunity to rat *neu* was detected and there remained uncertainty whether tolerance to rat *neu* was overcome (15). These results indicated that rat *neu* Tg mice may be somewhat inadequate for testing human ErbB-2 vaccines.

The development of human ErbB-2 Tg mice was previously attempted with a DNA sequence comprising the promoter-enhancer region of the MMTV-long terminal repeat and a constitutively activated allele of the human ErbB-2 (16). Expression of the transgene was observed in kidney, lung, mammary gland, salivary gland, and in the male reproductive track. All Tg mice expressing ErbB-2 died by 4 mo of age, probably due to kidney and lung failure following the development of preneoplastic lesions. Mammary glands in parous females were underdeveloped and some gave rise to tumors. Tg males were sterile.

To generate a new model of human ErbB-2 Tg mice, we have chosen the whey acidic protein (WAP) promoter. WAP is a major whey protein secreted in rodent's milk and WAP promoter has been used for targeting heterologous genes to the mammary gland. Although typically described as being under strict hormonal and lactational control, the expression of WAP transgenes in normal mice has also been observed in other organs, especially in the brain (17). Here we report the generation of Her-2 Tg mice using a WAP promoter regulated c-ErbB-2 transgene and the immune tolerance in these mice.

## Materials and Methods

### Construction of pWAP-human ErbB-2 (pWAP-Her-2)

The full-length human c-ErbB-2 cDNA was isolated from plasmid pCMV-ErbB-2 (9) as a 4.4-kb *EcoRI* restriction fragment and cloned into the *KpnI* site downstream of the 2.5-kb WAP promoter in pBSK kindly provided by

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Received for publication June 9, 2003. Accepted for publication September 30, 2003.

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<sup>1</sup> This work was supported by National Institutes of Health Grant CA76340; Department of Defense Grant ~~W5198-97-1-0007~~, National Institutes of Health Grants 5 T32 DC00026 (to M.P.P.) and P30 ES06639; and the Gail Puritan fund.

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<sup>3</sup> Abbreviations used in this paper: Tg, transgenic; WAP, whey acidic protein; MMTV, mouse mammary tumor virus.

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Dr. B. Groner (Chemotherapeutisches Forschungsinstitut, Frankfurt, Germany). *Escherichia coli* DH5 $\alpha$  (Life Technologies, Gaithersburg, MD) or Top10 (Invitrogen, Carlsbad, CA) was used to propagate pWAP-Her-2, using Terrific Broth (Life Technologies) containing 100  $\mu$ g/ml ampicillin. Plasmid was purified with QIAfilter Giga kit (Qiagen, Valencia, CA). The 6.9-kb WAP-Her-2 expression cassette was liberated by *Hind*III and used for transfection and microinjection.

#### Mice and cell lines

Male and female C57BL/6 (H2-K<sup>b</sup>) mice (6–8 wk of age) were obtained from Charles River Laboratory (Frederick, MD) or The Jackson Laboratory (Bar Harbor, ME). Mouse mammary tumor line D2F2 was derived from a spontaneous mammary tumor which arose in a BALB/c hyperplastic alveolar nodule line D2 (18). The cell line was maintained in vitro in DMEM supplemented with 5% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO) and 5% Cosmic Calf Serum (HyClone, Logan, UT), 10% NCTC 109 medium (Sigma-Aldrich), 2.5 mM 2-ME, 0.5 mM sodium pyruvate, 2 mM L-glutamate, 0.1 mM MEM nonessential amino acids, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. ID8/E2 is a C57BL/6 ovarian cancer cell line (19) transfected with pCMV-ErbB-2. EL-4/E2 is a C57BL/6 thymoma cell line transfected with pCMV-ErbB-2. All transfected cells were cloned twice by limiting dilution to isolate clones of stable expression. Transfected cell lines were maintained in medium containing 0.8 mg/ml G418 (Geneticin, Sigma-Aldrich).

#### Expression of WAP-Her-2 cassette in D2F2 cells

D2F2 cells were cotransfected with the 6.9-kb *Hind*III WAP-Her-2 expression cassette and linearized pRSV/neo, at a 10:1 ratio, using LipofectAMINE Plus reagent purchased from Life Technologies. Individual colonies were expanded and expression of the recombinant protein was analyzed by flow cytometry, or immunoprecipitation and Western blot. Some of the transfected D2F2 clones were cultured in the presence of 10  $\mu$ g/ml insulin and 10  $\mu$ M hydrocortisone to enhance transcription from WAP promoter.

#### Flow cytometric analysis

mAbs TA-1 (AB-5) and 3B5 (AB-3) which recognize the extracellular and cytoplasmic domains of ErbB-2, respectively, were purchased from Oncogene Research Products (Cambridge, MA). FITC conjugated goat anti-mouse IgG was the secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). Normal mouse Ig or isotype-matched mAb were the negative controls. Flow cytometric analysis was performed with a FACS-Calibur (BD Biosciences, Mountain View, CA).

#### Identification of Her-2 Tg mice by Southern blotting and PCR

For initial identification of Her-2 Tg mice, genomic DNA was isolated from the tail tissues of 3- to 4-wk-old mice using Qiagen genomic DNA isolation kit (Qiagen) and digested to completion with *Eco*RI (Life Technologies), transferred to charged nylon membranes in 20 $\times$  SSC, cross-linked to the membrane in a UV cross-linker and blocked overnight at 56°C in hybridization buffer (5 $\times$  SSC, 0.5% SDS, 5 $\times$  Denhardt's reagent, 1  $\mu$ g/ml BSA, and supplemented with 10  $\mu$ g/ml sheared salmon sperm DNA) in a Stratagene rotary hybridization oven.

Southern blot DNA probe was isolated from human ErbB-2 cDNA by *Eco*RI restriction (Fig. 1A). A 1.6-kb DNA fragment encoding ErbB-2 nt 1440–3000 was isolated and random primer labeled using the Klenow fragment of DNA polymerase to incorporate radioactive [<sup>32</sup>P]dCTP (DuPont NEN, Boston, MA; 3000 Ci/mmol). The reaction was stopped by the addition of EDTA and the product was purified by passage through a sephadex G-50 column which was prepacked and blocked with salmon sperm DNA (5 Prime $\rightarrow$ 3 Prime, Boulder, CO). Purified probe was denatured by boiling for 5 min and snap cooled on ice. Denatured probe was added to hybridization buffer to achieve a final concentration of  $2 \times 10^6$  cpm/ml. Specific activity of the probe was typically  $>10^8$  cpm/ $\mu$ g DNA. Membranes were hybridized overnight at 56°C and then washed to ultimate stringency (0.1 $\times$  SSC, 0.1% SDS, 65°C). Membranes were sealed in plastic wrap and exposed to Kodak MR-1 film with an intensifying screen for 2–3 days at –80°C.

For routine screening of Her-2 Tg mice, a 2-mm ear punch or tail tissue was collected from 3- to 4-wk-old pups and used for PCR analysis. The tissue was digested in 200  $\mu$ l of sterile lysis buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl<sub>2</sub>, 0.2 mg/ml gelatin (Sigma-Aldrich) and 0.45% v/v IGEPAL CA-630 (Sigma-Aldrich) supplemented with 100  $\mu$ g/ml proteinase K (Sigma-Aldrich P20308). The tissue was digested overnight at 56°C. Proteinase K was heat inactivated at 95°C for 30 min. The upper primer, 5'-CCC CCA CCC CAC CCC CAA AGT C-3',

anneals to the WAP promoter at position –22 relative to the ATG start codon. The lower primer, 5'-CGG GGG GCA AGA GGG CGA GGA G-3', anneals to the human ErbB-2 cDNA at aa 18 downstream of the signal peptide. Amplification of the transgene results in a 352 bp PCR product. Briefly, 2–5  $\mu$ l of genomic DNA was amplified in 1 $\times$  Qiagen reaction buffer, 200  $\mu$ M dNTPs, 1  $\mu$ M of each primer, and 1 $\times$  Q buffer in a total volume of 50  $\mu$ l with 1 U of *Taq* polymerase. After an initial denaturation at 94°C for 3 min, samples were amplified for 30–35 cycles, consisting of denaturing at 94°C for 1 min, annealing at 58°C for 1 min, followed by extension at 72°C for 1 min. After the last cycle, samples were incubated for 5 min at 72°C and resolved in 1.5% TAE-agarose gels.

#### Immunoprecipitation and Western blot analysis

Lysates from fresh tissues were prepared by mincing tissues (3 mm<sup>3</sup>) in prechilled 1.5 ml microcentrifuge tubes on ice in tissue lysis buffer (50 mM HEPES (pH 8.0), 10% glycerol, and 1% Triton X-100) supplemented with protease and phosphatase inhibitor mixtures (Oncogene Sciences, Cambridge, MA). Human ErbB-2 protein was immunoprecipitated from the tissue lysates by incubation with an anti-ErbB-2 mAb 4D5, or Herceptin (Genentech, South San Francisco, CA) or mAb 9G10.6 (Neomarkers, Fremont, CA) for 2–4 h. Immune complexes were recovered by incubation with protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 16–18 h. The agarose beads were subjected to centrifugation and washed twice with lysis buffer. Proteins were eluted in 1 $\times$  sample buffer and boiled for 3 min before fractionation in 6% SDS-PAGE. Proteins were electrotransferred to Immobilon-P (Millipore, Bedford, MA) polyvinylidene difluoride membranes. Membranes were fixed with methanol, rehydrated, and blocked overnight at 4°C in TBST buffer (10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1% Tween 20) with 1% BSA. ErbB-2 protein was detected by immunoblotting with mAb 3B5 or polyclonal C-18 (Santa Cruz Biotechnology). Phosphotyrosine was detected with mAb PY20 (Transduction Laboratories, Lexington, KY). Blots were developed with ECL reagents (Amersham, Arlington Heights, IL) and Kodak-MR film.

#### Immunohistochemical analysis

Tissues were removed from mice and placed immediately in phosphate buffered formalin. Paraffin sections were prepared at 4–5  $\mu$ m thickness and stained with H&E. For immunohistochemical analysis, endogenous peroxidase was blocked by incubation in 3% H<sub>2</sub>O<sub>2</sub> in methanol or Peroxoblock (Neomarkers). Mild treatment with ficin was used for epitope retrieval. Tissues were processed using the HISTOMOUSE SP kit from Zymed Laboratories (South San Francisco, CA) designed to stain mouse tissues with mouse mAbs. The primary Ab TAB250 (Zymed Laboratories) which recognizes an epitope in the extracellular domain of the human ErbB-2 protein was used according to the recommended procedure. Alternatively, paraffin sections were subjected to HIER (citrate pH 6.0) and stained with PAD: Z4881 specific for the intracellular domain of human ErbB-2 (Zymed Laboratories) followed by detection with anti-rabbit poly-HRP (Chemicon). Immunostaining was developed using DAB as the chromagen and nuclei were counterstained with hematoxylin. Sections were viewed under a Zeiss microscope equipped with a Sony 970 CCD camera and MCID5+ software interface for data acquisition and image analysis with the 25 $\times$  objective (100 $\times$  total magnification).

#### Testing tolerance and immunization of Her-2 Tg mice

For plasmid DNA immunization, mice from the eighth generation backcross were injected intramuscularly (i.m.) with 100  $\mu$ l of saline containing 100  $\mu$ g of each component plasmid at 2-wk intervals for a total of five vaccinations. Plasmid DNA expressing full-length and truncated variants of ErbB-2 gene have been described and characterized (9–11). The plasmid pEFBos-GM-CSF encoding murine GM-CSF was provided by Dr. Nishisaki (Osaka University, Osaka, Japan). At 2 wk after the final DNA vaccination, mice were challenged s.c. with  $2 \times 10^5$  EL-4/E2. Tumors were measured weekly with calipers and animals were sacrificed when any dimension of the tumor exceeded 15 mm. The percentage of tumor-free mice was analyzed by Kaplan-Meier method and statistical significance was determined by the log-rank test.

#### Measurement of anti-ErbB-2 Ab

Serum samples were diluted 1:20 and the presence of anti-ErbB2 Ab was determined by flow cytometry using SKBR-3 cells, a human breast carcinoma cell line with amplified ErbB-2. FITC conjugated goat anti-mouse Ab specific for mouse IgG Fc $\gamma$  (Jackson ImmunoResearch Laboratories) was used to detect bound primary Ab. Normal mouse serum or isotype-matched mAb was the negative control. The mAb TA-1 (Oncogene Research Products), which recognizes an extracellular domain of ErbB-2, was

used as a positive control for detection of ErbB-2 expression on SKBR-3 cells (Oncogene Research Products). Serial dilutions of TA-1 were used to generate a standard curve to determine the concentration (micrograms per milliliter) of anti-ErbB-2 Ab in serum. Flow cytometric analysis was performed with a FACSCalibur (BD Biosciences). Results are presented as concentration, or mean channel fluorescence. Statistical analysis was performed with Student's *t* test.

## Results

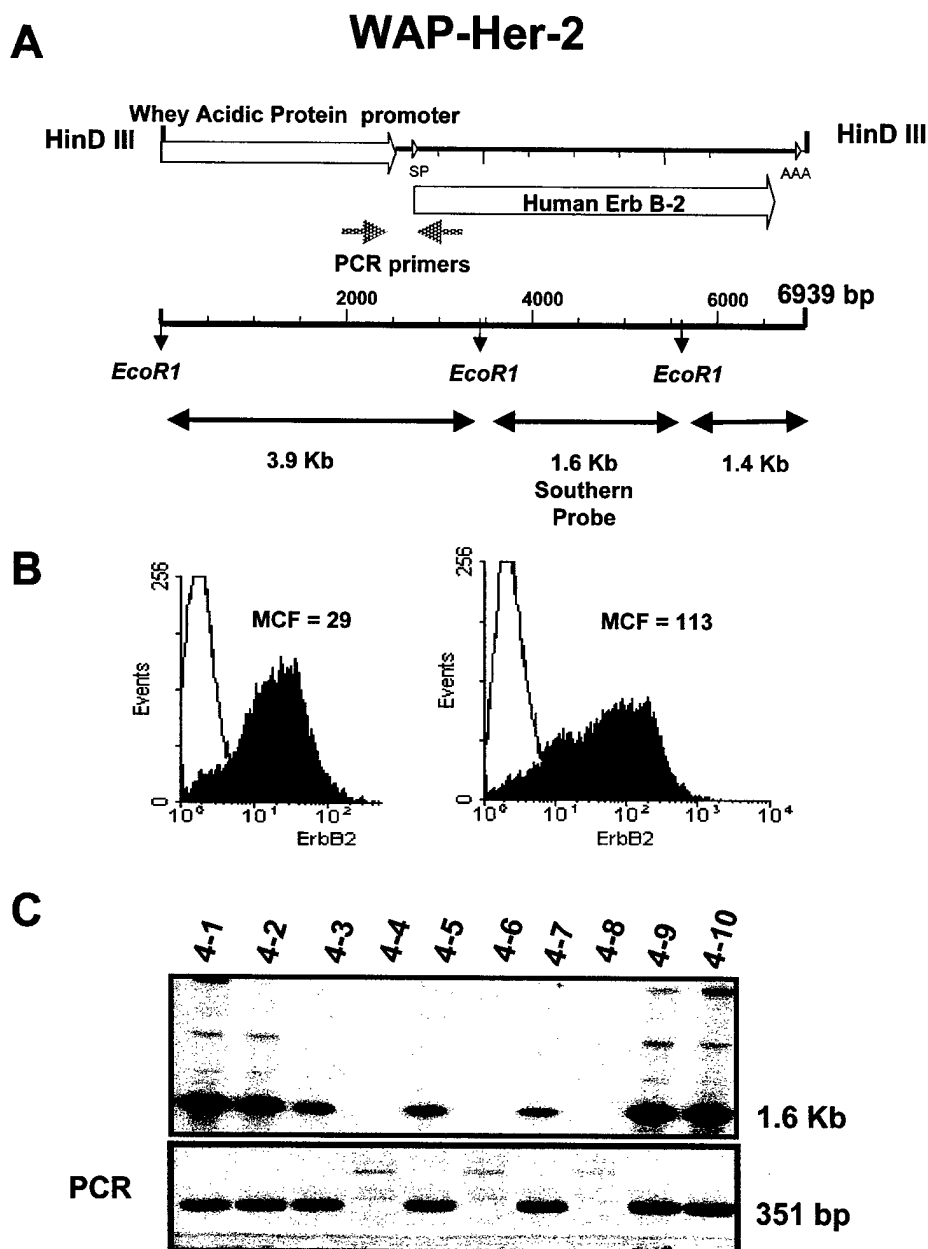
### Construction and expression of WAP-Her-2

WAP-Her-2 transgene was constructed by fusing the 2.6-kb mouse WAP promoter with the 4.4-kb human ErbB-2 cDNA as described in *Materials and Methods* (Fig. 1A). Expression of WAP-Her-2 cassette was initially tested by transfecting the 6939 bp fragment into mouse mammary tumor D2F2 cells. Human ErbB-2 protein was detected on the surface of transfected cells by flow cytometry (Fig. 1B). Treatment of the transfected cells with insulin and hydrocortisone resulted in nearly a 4-fold increase in ErbB-2 expres-

sion, indicating responsiveness of WAP promoter to the hormone. Immunoprecipitation and Western blot analysis verified the presence of the 185 kDa, phosphorylated human ErbB-2 (data not shown).

Purified WAP-Her-2 expression cassette was microinjected into fertilized eggs from B6C3 (C57BL/6 × C3H)<sub>F1</sub> females mated with B6C3 males following the procedure described by Hogan et al. (20). The embryos were implanted into pseudopregnant CD-1 surrogate mothers. From 97 live births, 7 positive mice were identified by Southern blot analysis using tail tissue lysates. These Tg founders were bred with C57BL/6 mice. A total of 60 pups were produced. Seven pups from 4 founders, carried the transgene as detected by Southern blot. These 7 pups were regarded as the *F*<sub>1</sub> founders and were mated with C57BL/6 mice to produce the first generation of backcross (*B*<sub>1</sub>) mice. Transgene-positive *B*<sub>1</sub> male mice were backcrossed with female C57BL/6 mice and transgene distribution in subsequent offspring followed Medelian rule. The

**FIGURE 1.** Construction of Her-2 transgene and genotypic identification of Her-2 Tg mice. **A**, A schematic representation of WAP regulated Human ErbB-2 (Her-2) transgene. The transgene is a 6939 bp linear *HinD*III fragment consisting of the 2.6-kb mouse WAP promoter fused with downstream 4.4 kb human ErbB-2 cDNA. Polyadenylation sequence at the 3' end of the gene are from the bovine growth hormone gene. The shaded arrows indicated the location of PCR primers used in genotyping. Probe for Southern blotting and the relevant *Eco*RI restriction sites are indicated. **B**, Expression of the human ErbB-2 transgene. D2F2 mouse mammary tumor cells were cotransfected with the WAP-Her-2 cassette and pRSV-neo. Stable clones expressing human ErbB-2 were selected and cultured in the absence (*left panel*) or presence (*right panel*) of 10  $\mu$ M hydrocortisone and 10  $\mu$ g/ml insulin for 48 h. Cell surface expression of human ErbB-2 was evaluated by flow cytometry with mAb TA-1 and detected with FITC conjugated goat anti-mouse secondary Ab (shaded histogram). The clear histogram represents binding of an isotype control Ab. **C**, Genotype analysis of Her-2 Tg mice. Positive identification of Tg animals is indicated by hybridization of the probe to a 1.6-kb *Eco*RI fragment (*top*) and by the amplification of a 352-bp PCR product (*bottom*). Southern blot and PCR analyses were performed on genomic DNA from the same 10 pups and the results demonstrated perfect concordance.



Tg mouse line which had the highest and most consistent Tg expression in the early generations was chosen to establish our colony. All studies described here were performed on the progeny of this line.

For routine screening of transgene expression, PCR primers were designed so that the upper primer annealed to the WAP promoter and the lower primer annealed to ErbB-2 cDNA. A 351-bp product was diagnostic of WAP-Her-2 gene. Using ear punch tissue from 10 pups of the first backcross ( $B_1$ ) generation, there was 100% concordance between Southern blotting and PCR analysis (Fig. 1C). Therefore, routine screening was performed with PCR.

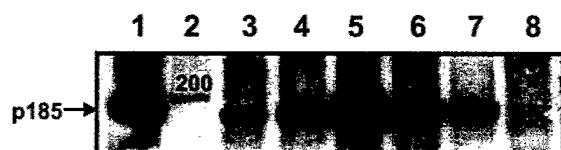
#### Tissue distribution of human ErbB-2 in Her-2 Tg mice

Human ErbB-2 expression in the mammary gland was examined in several Her-2 Tg females of the  $F_1$  (founder  $\times$  C57BL/6) generation (Fig. 2) at days 1–2 of lactation.

The no. 4 mammary glands were removed from Her-2 Tg females at 1–3 days after they delivered the first or second litter. Tissue lysates were prepared and immunoprecipitated with mAb Herceptin which recognized an epitope in the extracellular domain of human ErbB-2 and blotted with mAb 3B5 which recognized a carboxyl-terminal epitope. In Her-2 Tg mice from the  $F_1$  (lanes 4–5) generation, strong expression of p185 was detected. The same dominant band was detected in tissue lysate without immunoprecipitation (lane 3). Furthermore, this female produced two female pups with abundant human ErbB-2 expression in the mammary gland (lanes 6–7). No expression was detectable in the non-transgenic lactating littermates (lane 8).

Tg mice were crossed with C57BL/6 mice for 12 generations to establish Her-2 Tg mice in the C57BL/6 background. Mammary glands from Her-2 Tg females exhibit the HER2/Neu "signature" phenotype of branching mammary trees with extension beyond the normal fat pad and lobules arrayed in parallel along the milk line and the main artery that supplies all of the mammary glands.

Expression of human ErbB-2 was sustained in mice backcrossed with C57BL/6. In Fig. 3A, immunoprecipitation (IP) and Western blot was used to detect Human ErbB-2 gene product in the mammary tissue and cerebellum of  $B_{11}$  females on days 17–19 of pregnancy or, postvaginal plug release. Tissue lysates were immunoprecipitated with mAbs: 9G6.10, (Fig. 3A, lanes 2 and 4) or Herceptin (lanes 3 and 5) which recognized different epitopes of human ErbB-2. Immunoprecipitated proteins were detected by



**FIGURE 2.** Expression of ErbB-2 protein in Her-2 Tg mice. Immunoprecipitation and Western blot analysis of human ErbB-2 gene product in the mammary tissue of  $F_1$  females at 1–3 days postpartum. Mammary tissues were removed aseptically and placed in ice-cold lysis buffer. Tissues were minced and proteins immunoprecipitated with the anti-ErbB-2 humanized mAb, Herceptin. Proteins were resolved in SDS-PAGE, transferred to Immobilon-P, and hybridized with mAb 3B5 specific for a carboxyl-terminal epitope of ErbB-2. Bound Abs were detected with HRP conjugated goat anti-mouse Ig and visualized with chemiluminescent substrate. Lane 1 is a positive control of D2F2/E2 cells transfected with human ErbB-2. The arrow points to the 185-kDa human ErbB-2. Lane 2 is the molecular mass standards. Lanes 4–7 are proteins immunoprecipitated from mammary tissues of lactating transgene-positive females. Lanes 4 and 5 represent two  $F_1$  founders and lanes 6 and 7 are the offspring of the  $F_1$  in lane 5. Lane 8 shows proteins from the mammary tissues of a nontransgenic lactating female. Lane 3 is whole tissue lysate from the  $F_1$  in lane 5.

Western blot using pAb C-18 (Santa Cruz Biotechnology). Similar to  $F_1$  mice, p185 was detected in both mammary (lanes 2 and 3) and cerebellum (lanes 4 and 5) tissue lysates. Lane 1 is the cerebellum tissue lysate used for immunoprecipitation analysis in lanes 4 and 5. In Fig. 3B, we defined the localization of human ErbB-2 in the mammary gland (Fig. 3B, a and b) and cerebellum (Fig. 3B, c and d) using immunohistochemistry. Secretory mammary epithelia lining mature ductules and cross-sections of terminal endbud clusters exhibit intensely positive membrane staining by anti-human ErbB-2 Ab (arrows) while supporting stromal cells and fibroblasts are clearly negative (asterisks). In the cerebellum, intense ErbB-2 was detected in the molecular layer of the cerebellum (Fig. 3B, c and d). Distribution of the protein was prominent along the Bergman glia fibers (arrows, see figure legend) in the molecular layer (ML) and to a lesser extent in the membranes of the purkinje cells (PCL), but not detectable in the granular layer (GL). Transgene expression in the brain was also documented in WAP-hGH Tg mice (21).

ErbB-2 protein expression in Her-2 Tg males was also characterized. As in the females (Fig. 3B, c and d), intense human ErbB-2 specific immunoreactivity was observed in the molecular layer of the cerebellum in Her-2 Tg males (Fig. 3B, e and f). Human ErbB-2 protein was detected in the cerebellum by immunoprecipitation and Western Blotting (Fig. 3C, lane 3). Human ErbB-2 protein was not detected in the parotid (lane 1), other salivary glands (lane 2), or cerebrum (lane 4). The same tissues taken from a transgene-negative littermate were unequivocally negative (lanes 5–8). We further demonstrated that Tg ErbB-2 in the cerebellum was indeed phosphorylated on tyrosine residues using an HRP-conjugated mAb against phosphotyrosine (Fig. 3D) and verified the specificity using mAb P2NA that exclusively detects human ErbB-2 phosphorylated at tyrosine residue 1248 (not shown).

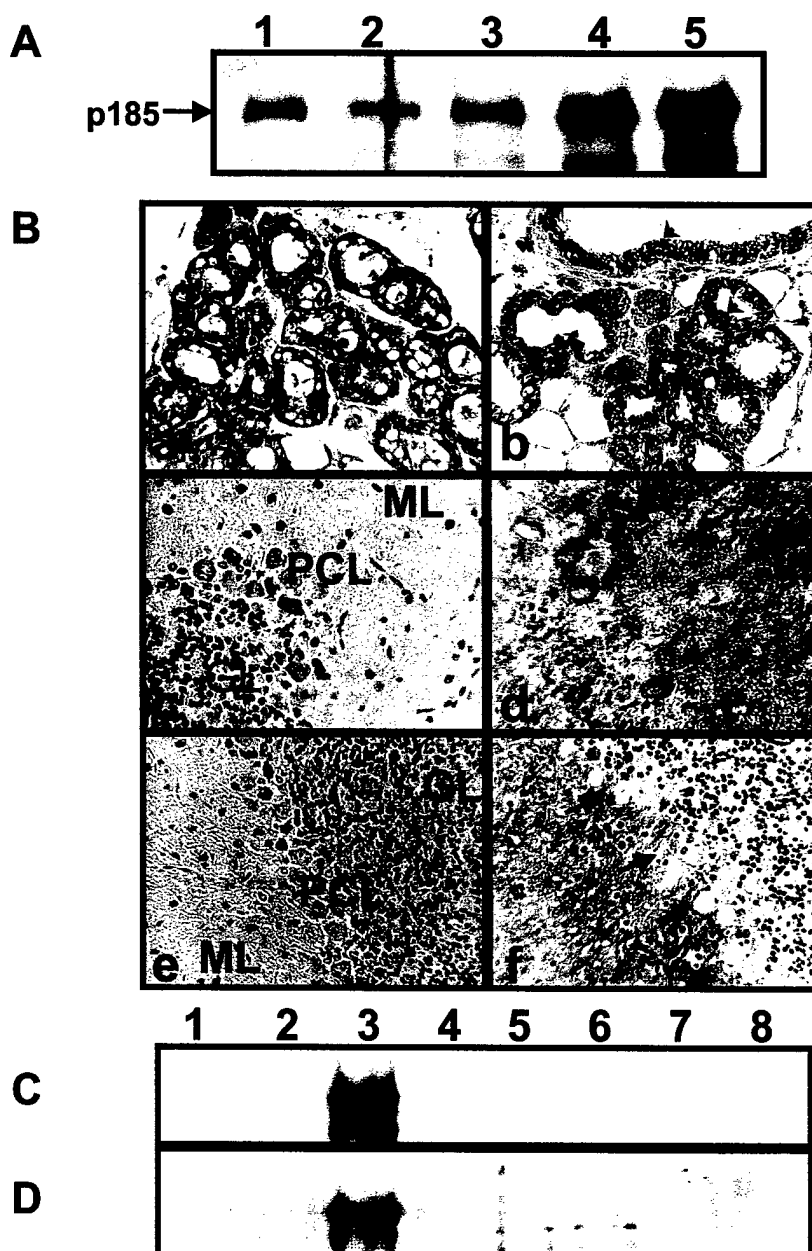
In males and females, human ErbB-2 protein distribution in the cerebellum was prominent in the Bergman glia cells, along their fibrous extensions and on the membranes of the purkinje cells which interface the molecular layer and the granular layer where the transgene is not expressed. This localization may indicate an association with the endogenous ErbB-3, which is expressed in the Bergman glia fibers and purkinje cells in adult cerebellum (22, 23). Other WAP-transgenes have been expressed in the brain, under hormonal regulation (24) or present in other regions of the brain (17). Constitutive ErbB-2 expression in the cerebellum appears unique to our Tg strain.

Compared with other organs tested, (including salivary gland, kidney, thymus, esophagus, adrenals, ovaries, and testes), ErbB-2 expression in the brain has been most consistent and at the highest level in Her-2 Tg mice from  $F_1$  to  $B_{12}$  generation independent of sex, lactational status, and parity (not shown). None of the tissues expressing human ErbB-2 demonstrated obvious abnormality. The mice were healthy and have a normal life span.

#### Immune tolerance to ErbB-2

To determine whether Her-2 Tg mice were tolerant to ErbB-2, growth of an immunogenic ErbB-2 bearing tumor was tested (Fig. 4). In normal C57BL/6 mice, injection of  $5 \times 10^6$  ID8 ovarian cancer cells resulted in tumor growth after ~2 mo (19). Transfection of ID8 with ErbB-2 (ID8/E2) increased the immunogenicity of the tumor and C57BL/6 mice rejected ID8/E2 tumor cells. Her-2 Tg mice injected with  $5 \times 10^6$  ID8/E2 cells began developing tumors after 5 mo and all mice were tumor positive by 9 mo (Fig. 4). Transgene-negative littermates did not develop ID8/E2 tumors ( $p < 0.01$  when compared with Her-2 Tg mice). Flow cytometric

**FIGURE 3.** Expression of ErbB-2 protein in Her-2 Tg mice fully backcrossed (11 generations) onto the C57BL/6 background. (A) Immunoprecipitation and Western blot analysis of human ErbB-2 gene product in the mammary tissue and cerebellum at lactation day (17–19). Tissues were minced and proteins were immunoprecipitated with mAbs specific for different epitopes of the human ErbB-2 protein: 9G6.10, (lanes 2 and 4) or Herceptin (lanes 3 and 5) in mammary (lanes 2 and 3) or cerebellum (lanes 4 and 5) tissue lysates. Proteins were detected using the polyclonal Ab C-18 (Santa Cruz Biotechnology). Lane 1 is a positive control cerebellum tissue lysate. The arrow points to the 185-kDa human ErbB-2. (B) Immunohistochemical localization of human ErbB-2 in the mammary gland (a and b) and cerebellum (c and d) of a lactating (days 17–19) Her-2-Tg female from the 11th backcross onto the C57BL/6 background. Secretory mammary epithelia lining mature ductules and cross-sections of terminal endbud clusters exhibit intensely positive membrane staining (arrows) while supporting stromal cells and fibroblasts are clearly negative (\*). These mature lobulo-alveolar structures are characterized by single layers of secretory epithelial cells with few adipocytes. In addition, the nonsecretory immature, undifferentiated mammary gland cells failed to demonstrate robust membrane ErbB-2 staining. Expression of human ErbB-2 in the cerebellum of Her-2 Tg female (c and d) and male (e and f) mice. Intense immunoreactivity using the TAB250 human-ErbB-2 specific mAb was observed in the molecular layer (ML) of the cerebellum (d and f). Distribution of the protein was prominent along the Bergman glial fibers (arrows) and to a lesser extent in the membranes of the purkinje cells (PCL), but not evident in the granular layer (GL). (C) Immunoprecipitation and Western blot analysis of human ErbB-2 gene product in salivary gland and brain tissues of male Her-2 Tg mice. Human ErbB-2 protein is exclusively expressed in the cerebellum of males from the 10th backcross (lane 3). No human ErbB-2 protein was detected in the cerebrum (lane 4) parotid (lane 1), other salivary glands (lane 2) nor in any of these corresponding tissues in transgene-negative littermates (lanes 5–8). (D) The immunoprecipitated human ErbB-2 in the cerebellum is phosphorylated on tyrosine residues as detected by the anti-phosphotyrosine mAb, PY20-HRP.



analysis of ascites tumor from Her-2 Tg mice showed ErbB-2 expression on the majority of cells. Therefore, there was not a selection for ErbB-2-negative ID8 cells in Her-2 Tg mice.

To examine the induction of anti-ErbB-2 Abs in Her-2 Tg mice, serum was collected 3 mo after ID8/E2 tumor cell injection. Anti-ErbB-2 Abs were not detected in Her-2 Tg mice while a significant induction of anti-ErbB-2 IgG Abs in transgene-negative littermates was detected ( $p < 0.05$ ) (Fig. 5). Abs against the parental ID8 tumor were not detected in either group (Fig. 5). These results indicated that Her-2 Tg mice were tolerant to ErbB-2 and this tolerance was not broken by the growth of an ErbB-2 overexpressing tumor.

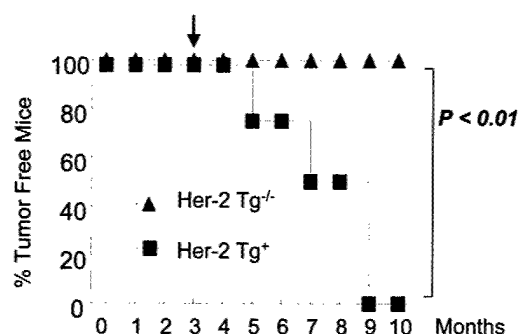
#### Anti-tumor immunity induced in Her-2 Tg mice by DNA vaccination

In a pilot study, we tested the induction of humoral immunity with pCMV-ErbB-2. After four i.m. injections with 100  $\mu$ g DNA, 2 wk apart, anti-ErbB-2 IgG was detected in 2 of 10 Her-2 Tg mice, whereas 3 of 3 transgene-negative littermates generated high levels

of anti-ErbB-2 specific Abs (not shown). To overcome tolerance in Her-2 Tg mice, we subjected Her-2 Tg mice and their transgene-negative littermates to a more robust DNA vaccination regimen. Her-2 Tg mice were covaccinated four times with pCMV secE2 encoding a secreted ErbB-2 extracellular domain and DNA encoding GM-CSF. Sera was collected one week after the fourth DNA vaccination and anti-ErbB-2 IgG Abs were measured as an indicator of anti-ErbB-2 immune response. Transgene-negative mice had significant anti-ErbB-2 IgG in their serum with an average of  $32 \pm 14$   $\mu$ g/ml (Fig. 6). Low level anti-ErbB-2 Abs were detected in 8 of 9 Her-2 Tg mice. One Her-2 Tg mouse had 43  $\mu$ g/ml anti-ErbB-2 IgG indicating that tolerance to ErbB-2 was clearly overcome in this mouse. Anti-ErbB-2 Abs were not detected in any unvaccinated Her-2 Tg mice.

To test the effect of vaccination on tumor growth, vaccinated mice were boosted once more and challenged 2 wk later with EL-4/E2 cells. All transgene-negative littermates were protected against EL-4/E2 challenge (Fig. 7). In contrast, only 33% of Her-2 Tg mice were protected. Using log rank test, the protection of



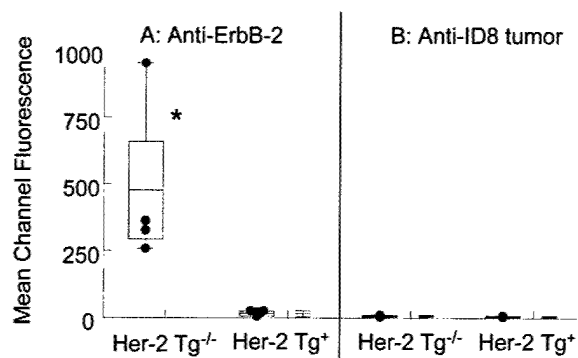


**FIGURE 4.** Her-2 Tg mice developed immunogenic ID8/E2 tumors. Her-2 Tg<sup>+</sup> ( $n = 4$ ) and Her-2 Tg<sup>-</sup> mice ( $n = 4$ ) from the eighth generation backcross, were injected i.p. with  $5 \times 10^6$  ID8/E2 cells in 0.5 ml PBS. Mice were palpated weekly for the onset of i.p. ascites. Mice were sacrificed when i.p. ascites were apparent. The arrow above the 3 mo interval denotes the time at which sera was sampled to determine levels of anti-ErbB-2 and anti-ID8 specific Abs (graphed in Fig. 5). Statistical significance was determined by the log-rank test.

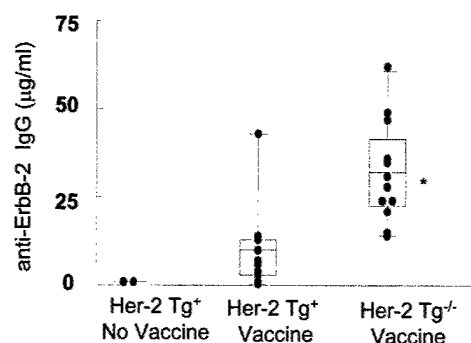
vaccinated Her-2 Tg mice was less than that of vaccinated transgene-negative littermates ( $p < 0.05$ ), but greater than that of control vector-injected Her-2 Tg mice ( $p < 0.05$ ). Therefore, five courses of i.m. injection with DNA encoding human ErbB-2 partially overcame humoral and possibly cellular tolerance to protect mice from tumor growth.

## Discussion

A human ErbB-2 Tg mouse strain (Her-2 Tg) syngeneic to C57BL/6 background has been established. Strong expression of human ErbB-2 was detected in lactationally active mammary epithelium and a high level of expression was detected constitutively in the brain of both male and female mice. Several other organs demonstrated variable expression. Her-2 Tg mice were tolerant to human ErbB-2 and permissive to the out-growth of tumors expressing human ErbB-2 without generating an Ab response. DNA vaccination in Her-2 Tg mice produced a modest but detectable anti-ErbB-2 Ab response and 33% of the animals were protected from tumor growth although 100% of the transgene-negative mice were protected. Induction of anti-ErbB-2 Ab was a sensitive indicator of immune response to ErbB-2 in the otherwise tolerant hosts, but



**FIGURE 5.** Anti-ErbB-2 Abs were not induced in Her-2 Tg mice. Her-2 Tg mice (Her-2 Tg<sup>+</sup>) and nontransgenic littermates (Her-2 Tg<sup>-</sup>) ( $n = 4$ ) were injected i.p. with  $5 \times 10^6$  ID8 cells transfected with ErbB-2. Three months later, serum was collected and used to stain SKBR-3 (A) or ID8 (B) cells. Bound Ab was detected by flow cytometry. The results are expressed as the mean channel fluorescence of individual samples. \*,  $p < 0.05$  by the Student's  $t$  test as compared with Ab production in Her-2 Tg mice.

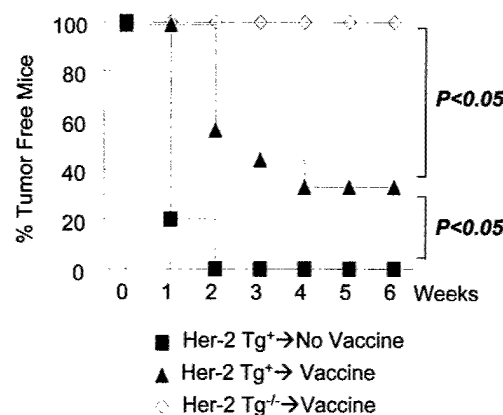


**FIGURE 6.** Induction of anti-ErbB-2 Abs in Her-2 Tg mice. Her-2 Tg mice ( $n = 9$ ) and negative littermates ( $n = 12$ ) from the eighth generation backcross, were immunized four times with 100  $\mu$ g each of pSecE2 and GM-CSF DNA. Control Her-2 Tg mice ( $n = 4$ ) were not vaccinated. Sera were collected after the final DNA vaccination and anti-ErbB-2 IgG Ab was measured by its binding to SKBR-3 cells and measured by flow cytometry. The results are expressed as micrograms per milliliter of individual samples. \*,  $p < 0.001$  by the Student's  $t$  test as compared with Her-2 Tg mice.

cellular immunity may contribute significantly to tumor rejection as Ab level did not correlate directly with tumor rejection.

These results are comparable to those found in *neu* Tg mouse models. Tg mice expressing either normal or activated rat *neu* demonstrated tolerance to *neu* Ag. In FVB Neu-N Tg mice expressing MMTV-*neu*, immunization with irradiated whole cell or recombinant vaccinia virus induced very weak cellular and humoral response when compared with FVB mice (25). In BALB NeuT mice expressing MMTV-NeuT, *neu* specific Ab and anti-tumor immunity was induced by vaccination with Neu DNA, although the level of response is much reduced when compared with transgene-negative littermates (26). Our findings demonstrate that tolerance in Her-2 Tg mice was partially overcome by DNA vaccination. This model enables us to test human ErbB-2 based vaccination strategies in a realistic, tolerant host.

Previous report demonstrated that MMTV-Her-2 Tg mice died within 4 mo of age, probably due to kidney and lung failure following the development of preneoplastic lesion (16). A viable



**FIGURE 7.** Anti-tumor immunity induced in Her-2 Tg mice. Her-2 Tg mice ( $\blacktriangle$ ) and nontransgenic littermates ( $\diamond$ ) from the eighth generation backcross, were immunized i.m. five times with 100  $\mu$ g each of pSec-E2 + GM-CSF. At 2 wk after the last immunization, mice were challenged s.c. with  $2 \times 10^5$  EL-4/E2 cells and included a group ( $n = 4$ ) Her-2 Tg mice that were not immunized to serve as positive controls for tumor growth ( $\blacksquare$ ). Tumor growth was measured by weekly palpation. Statistical significance was determined by the log-rank test.



Sprague-Dawley rat Tg for the wild-type human ErbB-2 gene has been described (27). In this model, expression of the Tg mRNA (under the control of the MMTV-long terminal repeat) was detectable in mid-pregnant but not virgin mammary tissues. After repeated cycles of pregnancy and lactation, pathological changes were produced in the mammary glands.

Using WAP promoter to express c-ErbB-2, Her-2 Tg mice developed normally without detectable lesions in the lung or kidney. ErbB-2 expression in the mammary gland was significant. The consistent and high level expression in the brain has been reported before with WAP promoter regulated transgenes. Human growth hormone driven by WAP promoter was highly expressed in the brain of both male and female Tg mice (21). Mice carrying WAP promoter regulated human urokinase-type plasminogen activator also demonstrated consistent transgene expression in the brain extract (28). Expression of human ErbB-2 in the brain did not have detectable pathological consequence in Her-2 Tg mice.

In normal human brain, ErbB-2 was detected consistently in oligodendrocytes, astrocytes and microglial cells and the level was elevated in patients with multiple sclerosis (29). ErbB-2 expressed in hypothalamic astrocytes mediates neuroendocrine functions. Luteinizing hormone-releasing hormone is released following stimulation of ErbB-2/4 complex on astrocytes by neuregulin, resulting in the development of female reproductive capacity (30). ErbB-2 is expressed in 86% of medulloblastoma, although it is not detected at any stage of cerebellar development, suggesting deregulation of ErbB-2 during medulloblastoma tumorigenesis (22). Cranial ganglia defects were detected in ErbB-2 knockout mice (31). ErbB-2 appears to play critical roles in normal and diseased brain. Further analysis of ErbB-2 in Her-2 Tg mouse brain may provide useful information. Vaccination of Her-2 Tg mice did not result in detectable autoimmune symptoms. Mice lived and reproduced normally. When Her-2 DNA vaccination efficacy is enhanced by increasing DNA uptake or modulating regulatory T cell, autoimmunity may be manifested and this should be closely monitored.

FVB NeuN mice crossed with C57BL/6 mice exhibit attenuated mammary tumor formation and increased tumor latency (32) and female mice develop tumors only after they experienced two to four pregnancies (33). The influence of strain background on mammary gland lesion incidence and phenotypes in WAP-TGF $\alpha$  Tgs has recently been described by Rose-Hellekant et al. (34). Here, we show that expression of WAP-ErbB-2 in C57BL/6 background resulted in a Her-2 tolerant mouse strain without spontaneous tumor growth. This strain will be valuable for testing ErbB-2 based vaccination against different tumor types. They can also be bred with mice expressing human HLA transgene to test human ErbB-2 vaccination in different HLA background. If spontaneous tumors are desired, Her-2 Tg mice can be bred with various oncogene Tg or tumor suppressor gene knockout mice to produce tumors of different nature.

## Acknowledgments

We thank Dr. Bernd Groner for his advice and support and David Shim and Kevin Tse for their excellent technical assistance.

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## Anti-tumor immunity and autoimmunity: a balancing act of regulatory T cells

Received: 5 June 2003 / Accepted: 7 August 2003 / Published online: 11 November 2003  
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**Abstract** Regulatory T (Treg) cell activity has been observed in anti-tumor and autoimmunity since the 1970s. Functional and molecular characterization of Treg cells has been made possible by the recent association of cell markers, such as CD25, CTLA-4, GITR, and Foxp3 gene product, with immunoregulatory activity. Here the influence of Treg cells in both anti-tumor immunity and autoimmunity was measured in BALB/c mice. Depletion of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells with CD25 mAb resulted in mammary tumor regression and increased susceptibility to thyroiditis. This in vivo priming to both tumor-associated antigens and self-thyroglobulin attests to the presence of otherwise undetectable immune effectors which are under negative regulation. Modulation of Treg cells is a powerful strategy in cancer therapy, but may potentiate autoimmune complications. Murine models exhibiting breakable tolerance to tumor-associated antigens, such as ErbB-2 (HER-2/*neu*), and increased susceptibility to autoimmunity following Treg-cell depletion are being established to test new vaccination or therapeutic strategies involving Treg-cell modulation.

### Introduction

The quest to control cancer by immunological means has met with only occasional success. There has been much speculation on this inadequacy, and many studies have been designed to delineate this difficulty [5, 15, 44]. For example, tumor cells may lose major histocompatibility antigen class I (MHC I), disabling their recogni-

tion by cytotoxic T cells [6, 44]. They may produce immunosuppressive molecules like TGF- $\beta$  [9, 32] to dampen immune stimulation. Solid tumors may be encapsulated, posing a physical barrier to immune cells [49]. To overcome these obstacles and elicit a functional anti-tumor immunity, a variety of strategies have been proposed. One of the most promising involves the modulation of regulatory T (Treg) cells which may inhibit the induction of anti-tumor immunity.

### Regulatory T (Treg) cells in anti-tumor immunity

Immunoregulatory or suppressor activity was observed in many laboratories in the 1970s [1, 3, 10]. Elaborate suppressor pathways were proposed to illustrate how suppressor cells and factors may inhibit anti-tumor immunity [40, 41]. It was suggested that tumor antigens preferentially activate the suppressor pathway [41], and that cyclophosphamide abrogates some, but not all, suppressive mechanisms [40]. At the time, the suppressor cells and factors were characterized mainly by their functions, since they were difficult to isolate and molecular markers were unavailable. Further studies regarding the nature of suppressor cells merely raised questions of their very existence, and the pursuit of suppressor cells was abandoned in most labs. With new molecular markers, suppressor-like or regulatory cells have been thrust into new light. Both myelocytic [27, 59] and lymphocytic regulatory cells have been described. The discussion here will be focused on Treg cells.

### Regulatory T (Treg) cells in autoimmunity

Similar to suppressor cells in anti-tumor immunity, suppressor cells in autoimmune diseases were described in the mid-1970s. In both the mouse [16] and rat [28], a low incidence of autoimmune thyroiditis arose spontaneously after postnatal thymectomy and irradiation to deplete putative suppressor T cells. A decade later,

This article forms part of the Symposium in Writing "Inhibitors of immunosurveillance and anti-tumor immunity," published in Vol. 53.

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cotransfer studies in nude mice showed a T-cell subset capable of suppressing the emergence of autoimmune diseases, such as gastritis, oophoritis, orchitis, as well as thyroiditis [37]. We demonstrated in a murine model of experimental autoimmune thyroiditis (EAT) that regulatory cells were induced in 2–3 days after tolerance induction with soluble mouse thyroglobulin (mTg) and that subsequent induction of EAT was prevented [18]. These regulatory cells were CD4<sup>+</sup> [19]. The rapid induction of regulatory activity suggested the amplification of an existing Treg population mediating natural tolerance. The recent identification of the CD25 (IL-2 receptor  $\alpha$ ) marker on cells with regulatory activity has permitted further characterization of Treg cells mediating natural tolerance. Whereas the transfer of CD25<sup>+</sup> cells into nude mice resulted in autoimmune diseases, including thyroiditis and gastritis, CD25<sup>+</sup> cells blocked such development [38]. Similarly, absence of CD4<sup>+</sup>CD25<sup>+</sup> Treg-cell activity led to autoimmune gastritis [42] and transfer of Treg cells delayed diabetes in CD28-deficient NOD mice [39]. The CD4<sup>+</sup>CD25<sup>+</sup> T cell was thus recognized as a candidate Treg cell in autoimmunity.

#### Characterization of Treg cells

Thymus-derived CD25<sup>+</sup> T cells represent ~10% of peripheral CD4<sup>+</sup> T cells and encompass a major Treg-cell activity. However, CD25 is expressed on both Treg and effector T cells. In vivo treatment of mice with a CD25 mAb, PC61, depletes Treg-cell activity in naive mice, but depletes both Treg and effector T cells in immunized mice. Other markers associated with CD4<sup>+</sup>CD25<sup>+</sup> Treg cells include CTLA-4 [34, 50], CD45RB<sup>low</sup> [12, 34], and toll-like receptors 4, 5, 7, and 8 [4]. Recently, two additional markers have shown high correlation with Treg activity. One is the glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) [26]. Similar to CD25, elevated GITR is expressed on both Treg and activated T cells [48]. GITR is a 228 a.a., cysteine rich, type 1 transmembrane protein of the TNF family [26] and forms homodimers on the cell surface. A nondepleting GITR mAb, DTA-1, triggers T cell signaling and abolishes suppressive activity without depleting cells [48]. Systemic treatment of BALB/c mice with GITR mAb resulted in spontaneous gastritis [48], indicating the induction of autoimmunity by functionally disabling Treg cells.

The most specific marker of Treg cells to date is scurf, a transcription factor encoded by forkhead box P3 gene *Foxp3* [7, 11, 14, 43]. Scurfin binds to the promoter region of cytokine genes and attenuates the production of activation-induced cytokine, such as IL-2 [43], keeping Treg cells from proliferating when stimulated. When transduced with *Foxp3* gene or isolated from *Foxp3* transgenic mice, even CD4<sup>+</sup>CD25<sup>+</sup> T cells exerted suppressive activity [11, 14]. Conversely, *Foxp3* knockout mice developed lymphoproliferative disease,

similar to *Foxp3*-mutant Scurfy mice or CTLA-4 [52] deficient mice, further supporting the association of *Foxp3* with negative regulation [7]. Overexpression of *Foxp3* in CTLA-4 deficient mice delayed lymphoproliferative disease. These findings support CD4<sup>+</sup>CD25<sup>+</sup>*Foxp3*<sup>+</sup> cells as a distinct lineage of Treg cells.

Functionally, Treg cells exert suppressive activity in a nonspecific fashion when their TCRs are triggered [51]. Cell-cell contact appears necessary [51], and membrane-bound TGF- $\beta$  may contribute to the activity [34]. This function may suppress self-reactive T lymphocytes that escape thymic clonal deletion [45, 46] or prevent overreaction to pathogens [36]. Other T cells may exist and regulate immune function through IL-10, but they may represent cell populations distinct from *Foxp3*<sup>+</sup> Treg cells [20].

#### Treg cell depletion on anti-tumor immunity and the induction of autoimmunity

Treg-like CD4<sup>+</sup>CD25<sup>+</sup> T cells with suppressive activity have been described in lung [58], pancreas, and breast cancer patients [22], although it is unclear if these cells are of *Foxp3* lineage. In mice, liberation from negative regulation by CD25 mAb treatment led to regression of leukemia and fibrosarcoma [47]. In another study, depletion of CD25<sup>+</sup> Treg cells resulted in lower incidence or slower growth rate of B16F10 tumor [13]. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells contributed to this anti-tumor activity. Priming to B16F10-associated antigens was evidenced by B16F10 tumor rejection in naive mice which received adoptively transferred immune CD4<sup>+</sup> T cells. Immune priming was further demonstrated by specific recognition of a self-antigen, tyrosinase, in mice which rejected B16F10 tumor with CD25 mAb treatment.

The amplification of anti-tumor immunity through Treg-cell modulation may be complicated by immune reactivity to non-tumor-associated, self-antigens. In 14 patients with metastatic melanoma, there were two complete and one partial responders following treatment with human mAbMDX-010 to block CTLA-4 engagement to B7, and s.c. vaccination with two modified HLA-A\*0201-restricted peptides [29]. In six patients (43%), grade III/IV autoimmune manifestations were observed, including dermatitis, enterocolitis, hepatitis, and hypophysitis. The three patients with objective cancer regression all developed severe autoimmune symptoms requiring treatment. The trial with a two-stage design was intended to accrue 21 patients in the first stage. The accrual was ceased after 14 patients because of these autoimmune complications.

By modulating Treg cells, cancer immunotherapy is encountering an unprecedented opportunity, yet one with a clear and present danger. Although autoimmunity is being handled in patients as it arises, there is an urgent need to analyze in a comprehensive manner the two sides of Treg-cell modulation. New and innovative

strategies must be developed to implement cancer control without falling prey to autoimmunity. Toward this end, animal model systems to assess simultaneously anti-tumor and autoimmunity following Treg-cell modulation have to be established.

## Results and discussion

### Treg-cell depletion on tumor growth in BALB/c mice

Many tumor models have been developed in BALB/c mice. Thus, the effect of Treg-cell depletion on tumor growth was tested in these mice using a D2F2/E2 tumor cell line. D2F2 is a mouse mammary tumor line derived from a spontaneous mammary tumor that arose in a BALB/c hyperplastic alveolar nodule (HAN) line D2, originally induced by prolactin stimulation [23, 57]. To introduce a defined tumor-associated antigen into the cell line, D2F2 cells were transfected with pCMV/E2 which encodes wild-type human ErbB-2 (HER-2) and a stable D2F2/E2 line was generated [57]. Human ErbB-2 (HER-2) is overexpressed in breast, ovarian, and several other cancer types. In stage IV breast cancer patients, therapeutic efficacy of HER-2 mAb, Herceptin, has been demonstrated. HER-2 based vaccines, including the HER-2 DNA vaccine generated in our lab, are being tested in clinical trials. In human ErbB-2 DNA immunized mice, rejection of D2F2/E2 tumor was mediated primarily by T cells, although significant humoral response was induced [31, 33].

BALB/c mice were injected i.p. with CD25 mAb on 2 consecutive days. Depletion of  $CD4^+CD25^+$  cells was verified by flow cytometry. On day 5 after the second mAb injection, when  $CD4^+CD25^+GITR^+$  T cells were at a minimum, mice were injected s.c. with  $2 \times 10^5$  D2F2/E2 cells. In untreated mice, D2F2/E2 tumors were palpable in 1 to 3 weeks after inoculation, and they grew to  $\sim 500 \text{ mm}^3$  in 6 weeks, when the mice were sacrificed (Fig. 1). Following Treg-cell depletion, six of eight mice

developed palpable tumors in less than 2 weeks, but the tumors started to regress before they were  $50 \text{ mm}^3$  in volume. By week 4, all tumors regressed completely. The mice were monitored for another 10 weeks without any sign of tumor recurrence. The same tumor regression was observed when mice received unmodified D2F2 tumor cells following CD25 mAb treatment (not shown).

The dramatic increase in anti-tumor immunity was induced by simply depleting Treg cells. This induction of functional immunity to a growing tumor (i.e., in situ priming) challenges the long-standing paradigm that solid tumors are poor at priming the immune system and cannot be rejected effectively by immune cells. Rather, their immunogenicity is thwarted by the regulatory pathway.

There is a finite window in which CD25 mAb can be used to selectively deplete Treg cells because activated T cells also express CD25 and are sensitive to this antibody. It would be detrimental if CD25 mAb is administered after effector T-cell activation. It may be possible to further amplify the striking anti-tumor immunity with reagents like GITR antibody which removes Treg-cell activity without depleting effector cells. These are powerful tools and there is significant concern that such treatment can open the gate to autoimmunity. To assess the extent of this possibility, we have chosen autoimmune thyroiditis as a prototype autoimmune disease to gauge the potential complications.

### Thyroiditis and Treg cells

Autoimmune thyroiditis was among the first autoimmune complications recognized in the mid-1970s, following the removal of putative suppressor T cells in rodents [16, 28]. Because experimental autoimmune thyroiditis (EAT) is inducible with a self-antigen, mouse thyroglobulin (mTg), a homologue of human thyroglobulin, and shares well-delineated features with human Hashimoto's thyroiditis (HT), it has served as a prototype autoimmune disease model [17, 55]. HT is a well-characterized, organ-specific disease with known thyroid antigens. Similar to EAT with susceptibility linked to the mouse MHC class II genes, HT susceptibility is strongly influenced by the human MHC class II genes, such as HLA-DRB1\*0301, supplemented by CTLA-4 genes in disease development [53, 54]. Clinically, HT is an easily diagnosed hypothyroid syndrome, characterized by elevated thyroid-stimulating hormone (TSH) and decreased thyroid hormone levels. This syndrome results from destruction of the thyroid gland by mononuclear cell infiltration [56]. Early autoimmune responses can be monitored by autoantibody production and T-cell proliferative response in vitro to thyroid antigens. Although HT progresses chronically, it can be managed by thyroid hormone replacement therapy.

The role of Treg cells in EAT tolerance has long been recognized. Treg activity in EAT can be specifically enhanced by elevating the circulatory mTg level for

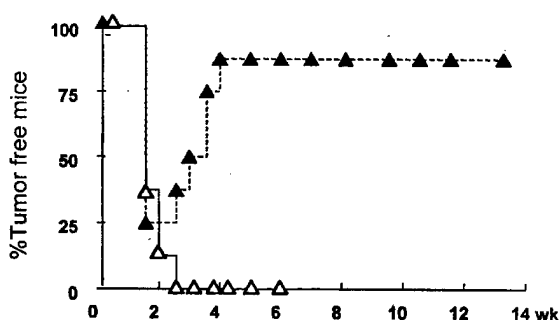


Fig. 1 Tumor regression following  $CD4^+CD25^+$  T-cell deletion. BALB/c mice were injected i.p. with CD25 mAb PC61 (solid triangle) 5 and 6 days before they received s.c.  $2 \times 10^5$  D2F2/E2 cells. Control mice were treated with normal mouse sera or PBS (open triangle). There were eight mice each in PC61 treated and control groups

2–3 days, either by pretreating susceptible mice with mTg [18], as mentioned above, or by TSH infusion to release endogenous mTg [21]. Subsequent induction of EAT with mTg and adjuvant such as complete Freund's adjuvant or lipopolysaccharide is markedly suppressed in such tolerized mice. Tolerance is mediated by CD4<sup>+</sup> Treg cells [19] which exert their suppressive action for >10 weeks [8]. The interval of 2–3 days for TCR engagement with elevated mTg antigen is sufficient for the activation and expansion of Treg cells, such that the animals can withstand EAT induction with mTg plus an adjuvant. It is important to note that immunization with mTg plus proinflammatory cytokines, such as IL-1 [25] or IL-12 [60], was not sufficient to overcome this tolerant state. We have ruled out T<sub>H</sub>2-related influence on Treg activity, since IL-4 and IL-10, separately or together, did not participate in mTg-induced tolerance [61].

#### Treg-cell depletion on thyroiditis

The potential of using EAT as a prototypical autoimmune disease to assess the effect of Treg-cell manipulation was tested by depleting Treg cells in tolerized mice prior to EAT induction. EAT-susceptible CBA mice were used initially. Mice received the tolerogenic regimen of two doses of deaggregated mTg (dmTg). Treg cells were then depleted with two doses of CD25 mAb, 4 days apart. The mice were subsequently challenged with the EAT-inducing regimen of mTg and LPS. Depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells abrogated protection against EAT induction; 100% (6/6) of tolerized mice became susceptible to EAT induction with thyroid damage (thyroid infiltration of 10% or more), compared with 17% (1/6) mice in the tolerized, IgG control group (Fig. 2).

With the establishment that Treg cells are involved in the immune regulation of EAT-susceptible CBA mice [24], the role of Treg cells was tested in a relatively

EAT-resistant BALB/c strain. In a pilot experiment, anti-CD25 treatment prior to EAT induction led to marked thyroid damage in 100% of the mice, compared with mild inflammation in 43% of control mice (data not shown). Therefore, Treg cells are critical in maintaining EAT resistance in BALB/c mice and the effect of Treg-cell modulation on autoimmune thyroiditis can be assessed in these mice.

#### Tolerance to tumor-associated antigens

The pressing need to modulate Treg cells stems from the observed tolerance to tumor-associated antigens. Although profound anti-tumor immunity to tumors like D2F2/E2, which contains a foreign human ErbB-2 antigen, can be readily induced in BALB/c mice by one-time, transient depletion of Treg cells (Fig. 1), the same may not be true for an overexpressed self-antigen in human cancer. More strenuous Treg-cell modulation may be required to achieve therapeutic effect, creating an even greater risk of autoimmunity [29]. A test system with tolerance to tumor-associated antigen, e.g., ErbB-2, is needed to assess the effect of Treg-cell modulation. BALB NeuT (NeuT) mice are suited for this purpose. NeuT mice express a transforming rat Neu under MMTV promoter and have been backcrossed to BALB/c mice for >12 generations [2]. NeuT females develop atypical hyperplasia, carcinoma in situ, and palpable mammary tumors at ~5, 10, and 17 weeks of age, respectively. Males develop salivary tumors at ~7 months of age. Spontaneous tumors express abundant transgenic Neu. These NeuT mice are tolerant to Neu and respond weakly to Neu vaccination [35]. Blockade of spontaneous tumorigenesis in NeuT mice is a significant challenge, particularly after carcinoma has been well established in situ.

In our lab, transgenic mice expressing wild-type human ErbB-2 (HER-2 Tg) have been established [30] and have been backcrossed with C57BL/6 mice for >12 generations. These mice do not develop spontaneous tumor, but are tolerant to human ErbB-2. It may be fruitful to test the effect of Treg-cell modulation on ErbB-2-positive tumor growth and to monitor autoimmune complications in BALB NeuT or C57BL/6 HER-2 Tg mice.

#### Balancing anti-tumor and autoimmunity when manipulating Treg cells

New strategies to induce strong anti-tumor immunity without significant autoimmunity will be the next milestone in tumor immunotherapy. To achieve anti-tumor immunity, it will be important to eliminate Treg cells to tumor-associated antigen. To avoid autoimmunity, it is advantageous to amplify Treg cells to self-antigens. The available tools for manipulating Treg cells, such as mAbs to CD25, GITR, and CTLA-4, do not distinguish

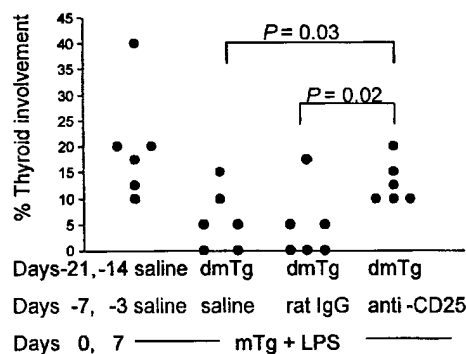


Fig. 2 In vivo depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells in mice with established tolerance abrogates protection against EAT. Mice were given 100-μg deaggregated mTg or saline, followed by CD25 or IgG control as shown. EAT was induced with 40-μg mTg + 20-μg LPS, and thyroid pathology was examined on day 28

one Treg cell from another. Will these tools be useful in cancer therapy? Perhaps the first order of business is to determine whether all Treg cells in a given individual are created equal and are therefore equally sensitive to these modulating agents. Using thyroiditis as a model system with known MHC class II-based susceptibility, one can test if Treg cells generated during thyroglobulin-induced tolerance are qualitatively different from resident Treg cells in na mice. Alternatively, it may be advantageous to inactivate tumor-specific Treg cells locally at the vaccination site to amplify priming to tumor-associated antigens without systemic down-modulation of Treg cells, thus minimizing the priming to self-antigens. The insight on Treg cell biology, the available molecular tools, and the awareness of autoimmune complications will be critical in cancer immunotherapy.

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